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In-vitro and *in-vivo* Evaluation of Poly (Propyl Ether Imine) (PETIM) Dendrimer for Sustained Delivery of Zidovudine

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Abstract

Study background: Acquired immunodeficiency syndrome (AIDS) is a severe disease of immune system caused by Human Immunodeficiency Virus (HIV). Zidovudine (AZT) is an effective antiretroviral drug against HIV. Due to its poor bioavailability (50-60%) and short elimination half life (0.5-3 h), frequent administration of zidovudine is required resulting into dose dependent hematological toxicity. In the present study, an attempt was made to develop sustained release formulation of AZT employing the poly (propyl ether imine) (PETIM) dendrimer as a carrier.

Methods: The AZT-dendrimer complex was prepared and studied by Fourier transform infrared (FTIR) analysis and nuclear magnetic resonance (NMR) spectroscopy. The entrapment efficiency and *in-vitro* drug release studies were conducted by dialysis bag method. Hemolytic toxicity, pharmacokinetic and biodistribution studies were performed to evaluate the biosafety and sustained release characteristic of prepared formulation.

Results and Conclusion: The cumulative amount of zidovudine released in 1 h from the AZT-dendrimer formulation was 6.5 ± 0.3 % compared with 95.8 ± 4.1% from the control drug solution. Zidovudine release was prolonged upto 14 h with zidovudine-dendrimer complex (94.3 ± 3.8%). The findings of present investigation illustrated that PETIM dendrimer effectively encapsulate AZT, and can be used as a biosafe carrier for sustained delivery of zidovudine.

Keywords: AIDS; HIV; Zidovudine; Dendrimer; Sustained release; *in vivo* pharmacokinetic and biodistribution studies

Introduction

Acquired immunodeficiency syndrome (AIDS) is a disease of the human immune system caused by infection with human Immunodeficiency Virus (HIV) [1]. Since the early reports of AIDS epidemic, it has become a harrowing disease which has spread like a plague. According to UNAIDS, 1.6 million people died globally in 2012 due to AIDS, while 35.3 million people are living with HIV out of which 2.3 million cases alone appeared in 2012 [2]. So these alarming figures are sufficient to depict the challenges associated with the cure and prevention of the disease. Still no single drug or method is available which can completely eradicate the HIV and cure the AIDS. However, number of policies has appeared like Millennium Development Goal (MDG 6), National HIV/AIDS Strategy (NHAS), International HIV/AIDS Alliance and presently, lot is being done to manage AIDS which resulted in significant reduction in the number AIDS cases over the past few years.

Zidovudine (AZT) is the first antiretroviral approved by FDA for the treatment of AIDS [3]. It significantly reduces the replication of the virus in patients leading to clinical and immunologic improvements [4]. However, the systemic availability of AZT after oral administration is only 50-60%, following first-pass metabolism. The biologic half life of AZT is 0.5 to 3.0 h, which requires frequent administration of the drug. The main limitation of AZT is that it is known to cause dose dependent hematological toxicity leading to anemia and leucopenia [5,6]. It is crucial for the success of AIDS therapy to maintain the systemic drug concentration consistently above its target antiretroviral concentration throughout the course of the treatment [7,8]. So sustained drug delivery systems for AZT seems reasonable, which can effectively deliver the drug maintaining the constant therapeutic AZT concentration over a prolonged period of time, and that too well below its concentration causing hematological toxicity. Wannachaiyasit et al. [9] studied the conjugation of AZT with dextrin and found improved the pharmacokinetics of AZT in rats by providing significant greater area under the plasma concentration-time curve and reduced the systemic clearance of AZT compared to free AZT [9].

The aim of the present investigation is to develop carrier based AZT formulation for its sustained delivery with objectives of maintenance of constant plasma concentration within the therapeutic range for longer periods, reduce the frequency and severity of side effects of AZT, which generally occurs immediately after conventional intravenous or oral administration. In our previous study, we have successfully employed PETIM dendrimer as a carrier for sustained delivery of ketoprofen [10]. The PETIM dendrimer was evaluated for its acute and short term sub-acute toxicity profile by studying the effect of PETIM administration on hematological, biochemical parameters of mice. Results depicted that PETIM dendrimer is biocompatible and safe to use for drug delivery applications. In the present investigation, we have prepared AZT loaded poly propyl ether imine (PETIM) dendrimer for its sustained drug delivery. The developed AZT-dendrimer complex is characterized extensively both *in-vitro* and *in-vivo*.

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Materials and Methods

Materials

Zidovudine was received as a gift sample from Cipla Ltd., Mumbai. Dialysis membrane having molecular weight cut off 1 kD was purchased from Spectrapor, USA. Acetonitrile was purchased from Loba Chemie Pvt. Ltd., Mumbai, India. All other reagents used were of analytical grade.

Development and *in-vitro* characterization of AZT-dendrimer complex

PETIM dendrimer was synthesized using the previously described method [11-13]. AZT-dendrimer complex was prepared as per method reported by Papagiannaros et al. [14]. AZT was dissolved in methanol and synthesized PETIM dendrimer was added. This solution was stirred overnight by magnetic stirrer (50 rpm), then dried under vaccum to remove methanol (Vaccum oven, Perfit, India). The complex was dissolved in distilled water and stirred for another 2 h to extract out the drug-dendrimer complex. The solution was then filtered through the 0.22 µm membrane filter and lyophilized (Heto, Allerod, Denmark) to remove the water completely. The drug-dendrimer complex was redissolved in PBS (7.4) and used for *in-vitro* and *in-vivo* characterization.

Characterization of drug-dendrimer complex was carried out by recording the FTIR and ^{1}H NMR spectra. FTIR spectra of AZT, AZT-dendrimer complex and dendrimer alone was recorded using Perkin Elmer, Spectrum RX-I, IR Spectrophotometer. ^{1}H NMR spectra of AZT, AZT-dendrimer complex and dendrimer alone was recorded with deuterated water (D_2O) as solvent using Bruker, Advance II 400 NMR spectrometer. Clarity of drug-dendrimer complex was determined by visual inspection. Encapsulation efficiency was determined by separating free drug by dialysis for 4 h, followed by determination of drug content by HPLC assay.

In-vitro drug release study

In-vitro release behavior of zidovudine from the AZT-dendrimer complex was investigated by dialysis method as described by Wang et al. [15]. Pure zidovudine was dissolved in PBS (pH 7.4) at a concentration of 2 mg/ml and used as control. The complex was also dissolved in PBS (pH 7.4) at a concentration of 2 mg/ml. This solution (2 ml in volume) was transferred to dialysis bag (molecular weight cut off 1 kD) immediately. The dialysis bag was placed in a 50 ml-beaker containing 40 ml PBS (pH 7.4). The outer phase was stirred continuously using magnetic stirrer at 50 rpm. At predetermined time intervals of 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 15, 20 and 24 h, Sample (1.0 ml) was withdrawn and replenished with the same amount of receptor fluid. The concentration of zidovudine present in the dialysate was monitored using HPLC and the percent drug release at various time intervals was calculated.

Hemolytic toxicity of AZT-dendrimer complex

The RBC suspension was obtained as per method reported for hemolytic studies [16]. The RBC suspension was mixed with distilled water, which was considered as producing 100% hemolysis, and normal saline producing no hemolysis, thus, acting as blank. 0.5 ml of RBC suspension was added to 4.5 ml of PBS (pH 7.4), and then 20 µg/ml, 40 µg/ml, 60 µg/ml of dendrimer solution, AZT solution and AZT-dendrimer complex were interacted with RBC suspension. After 1 h incubation at 37 ± 1.0°C, followed by centrifugation, supernatants were taken and diluted with an equal volume of PBS (pH 7.4) and absorbance was taken at 540 nm against supernatant of normal saline diluted similarly as blank. The percent hemolysis was thus determined for each sample by taking absorbance of water as 100% hemolytic sample.

Cytotoxicity assay

Freshly thawed, actively growing small lung cancer cell line (A-549) was used in cytotoxicity assay. Twenty hours old, freshly seeded, actively growing cultures of the selected cell lines were harvested by trypsinization/shaking and centrifuged at 3000 rpm for 5 min. The cells were then washed with PBS and resuspended in RPMI containing 10% FBS at a concentration of 10^6 cells/ml. Microtiter plates were seeded by transferring 100 ml cells to each well and incubated at 37 ± 0.5°C for 24 h under 5% CO_2 atmosphere (5% CO_2 incubator, New Brunswick Scientific, Ltd. Germany). The final number of cells seeded in each well was 5000. A stock solution (100 ml) was added in first well of the 96 well microtiter plate (B.D. Bioscience Ltd., USA), followed by serial dilution by 10X in subsequent well. The plate was incubated for 24 h and cell viability study was conducted using MTT assay. MTT solution (0.5% w/v in RPMI medium) was added to each well, and the plate was incubated at 37 ± 0.5°C for 4 h. After 4 h, the content of each cell was removed and the converted dye was solubilized in 150 µl isopropyl alcohol/dimethyl sulfoxide mixture (1:1). Absorbance of converted dye was measured at wavelength of 540 nm using ELISA plate reader (B.D. Bioscience Ltd., USA).

Pharmacokinetic and biodistribution studies

Albino rats (100-200 g in weight), Sprague-Dawley strain, 6 to 8 weeks old was used to study the biodistribution of AZT-Dendrimer complex. Rats were divided into two groups each having six animals. First group received AZT solution in PBS (pH 7.4) and second group were administered AZT-Dendrimer formulation (in PBS (pH 7.4)) in equal doses intra-peritoneally. After 6 and 12 h, animals from each group were sacrificed and the organs (liver, kidney, lymph nodes and spleen) were excised and homogenized in PBS. The homogenates were extracted, deproteinized with acetonitrile, then centrifuged, filtered and estimated for drug content using HPLC assay, as described below. Same animals were used for pharmacokinetic and biodistribution studies.

Estimation of drug in plasma

Blood was collected from cardiac puncture in a centrifuge tube containing heparin sodium as anticoagulant and centrifuged at 2000 rpm for 15 minutes (Remi Equipment, Mumbai, India), supernatant was collected and acetonitrile (1 mg/ml) was added to precipitate the proteins. The precipitated proteins were settled by centrifugation at 2000 rpm for 15 min and collected the supernatant. One ml of collected supernatant was filtered through a 0.45 µm membrane filter and collected into a 10 ml volumeteric flask, volume was made up with mobile phase and drug concentration was determined by HPLC assay.

Estimation of drug in various organs

Various organs (liver, spleen, kidney and heart), after drying in tissue paper, were weighed and minced into small pieces. One gram of each organ was homogenized with 2.0 ml of PBS (pH 7.4). In the organs weighing less than one gram, whole organ was used. To tissue homogenate, 2 ml acetonitrile was added and kept for 30 minutes. The resultant suspension was centrifuged for 20 min. at 5000 RPM,

filtered through a 0.45 µm membrane filter and collected into a 10 ml volumetric flask, and volume was made up with mobile phase, drug concentration was determined by HPLC assay and % dose recovered in comparison to plasma concentration of drug was calculated.

HPLC assay

In the present study, AZT was estimated by the HPLC method. Acetonitrile and distilled water (75:25) was used as mobile phase and flow rate was set at 1.0 ml/min. The injected fluid (20 µl) was eluted in C18 column (Agilent Technologies, USA 250×4.6 mm) at room temperature, and zidovudine was monitored at 267 nm using a UV detector (Agilent Technologies, USA). Observed chromatogram of AZT is shown in Figure 1. AZT was eluted forming well shaped, symmetric single peaks, well separated from the solvent front with retention time of 4.4 min.

Statistical analysis

Data are expressed as the Mean ± Standard Deviation (SD) of obtained results. The statistical analysis of data was performed using analysis of variance (ANOVA) (Graphpad, Version 2.01, San Diego, CA). A value of $p<0.05$ was considered as statistically significant.

Results and Discussion

Preparation and *in-vitro* characterization of AZT-dendrimer complex

Table 1 shows the composition of AZT-dendrimer complex. AZT-dendrimer complex was prepared by reported stirring method [14]. Initially, drug-dendrimer complex was prepared with three molar ration of AZT:dendrimer 10:1, 5:1 and 3:1 and optimized with respect to entrapment efficiency and results of *in vitro* drug release study of complex. The % entrapment efficiency of AZT–dendrimer complex (3:1 molar ratio) was found to be highest of 70.0 ± 4.2%. The probable reason for higher entrapment efficiency of AZT is due to higher internal cavity diameter (14.14°A) [17] of PETIM dendrimer, and low molecular weight of AZT (267 g/mol) leads to effective encapsulation of AZT in internal cavity of dendrimer.

The prepared AZT-dendrimer complex was characterized by determining the IR and NMR spectra of AZT, PETIM dendrimer alone and AZT-dendrimer complex, as shown in Figures 2A-2C and 3A-3C. In the FTIR spectrum A, the band at 1686 cm^{-1} corresponds to the stretching vibration of the C=O group of AZT. The stretching vibrations at 3486 cm^{-1} corresponds to the OH and NH group, and the peaks from 2813 to 3160 cm^{-1} represents the stretching vibrations of CH groups (sp^3 and sp^2 hybridized). The bands at 1260 and 1089 cm^{-1} represents the C-O and C-N stretching vibration. The stretching vibration at 2082-2117 cm^{-1} corresponds to the azide group. The characteristic bands of the dendrimer were also observed in the FTIR spectrum (Figure 2B) of the dendrimer with bands at around 3350, 2850-2970, 1500 and 1200 cm^{-1} exhibiting the presence of OH, CH, C=O and C-O functional groups, respectively. The FTIR spectra (Figure 2C) of drug-dendrimer complex showed that characteristic carbonyl group peak of AZT with almost the same intensity. However, this band was observed as splitted band. This is due to formation of drug dendrimer complex. The shape of the band due to OH group (sharp in case of AZT) in the drug dendrimer complex changed from sharp to broad which might be due to merging of OH, NH stretches of the drug and the dendrimer. The dendrimer may complex the drug via various mechanisms, i.e. dipole-dipole interaction, hydrophobic interaction, hydrogen bonding or electrostatic attraction [18]. Similarly, ^{1}H NMR spectra showed the formation of drug dendrimer complex (Figure 3).

In-vitro drug release study

The AZT release rate from drug-dendrimer complex is an important parameter, since a sustained release is necessary in order to decrease the dose dependent side effects of AZT and improve its therapeutic index. Figure 4 shows the % amount of drug released from the AZT-dendrimer complex and plain drug. Plain drug was taken as control to determine the sustained release potential of developed formulation. The % amount of AZT released in 1 h from the AZT-PETIM complex

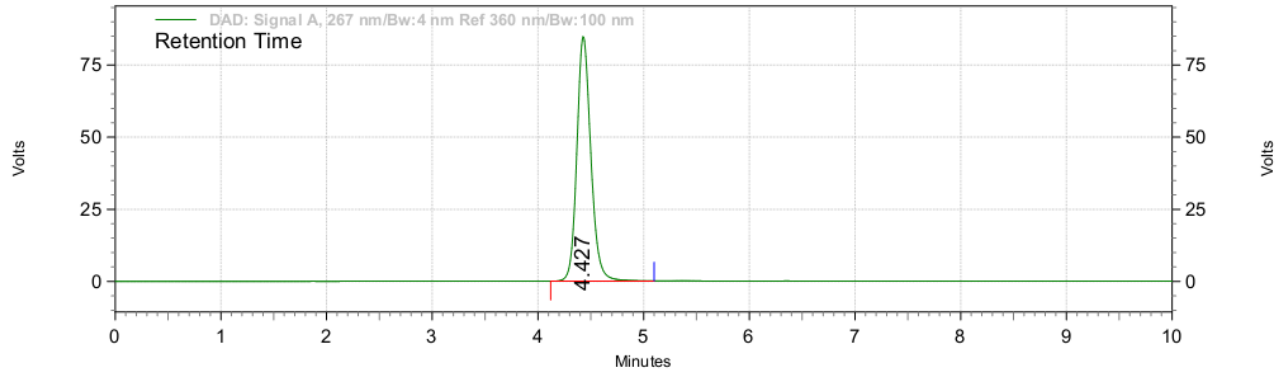

Figure 1: HPLC chromatogram of AZT.

Formulation code	Dendrimer (Mole)	Drug (Mole)	Physical appearance	Encapsulation efficiency
Den-AZT_1	1	3	Clear solution	70.0 ± 4.2%
Den-AZT_2	1	5	Clear solution	52.1 ± 2.8
Den-AZT_3	1	10	Clear solution	39.4 ± 2.1

Data are represented as Mean ± SD (n=3)

Table 1: Composition and characterization of AZT-dendrimer complex.

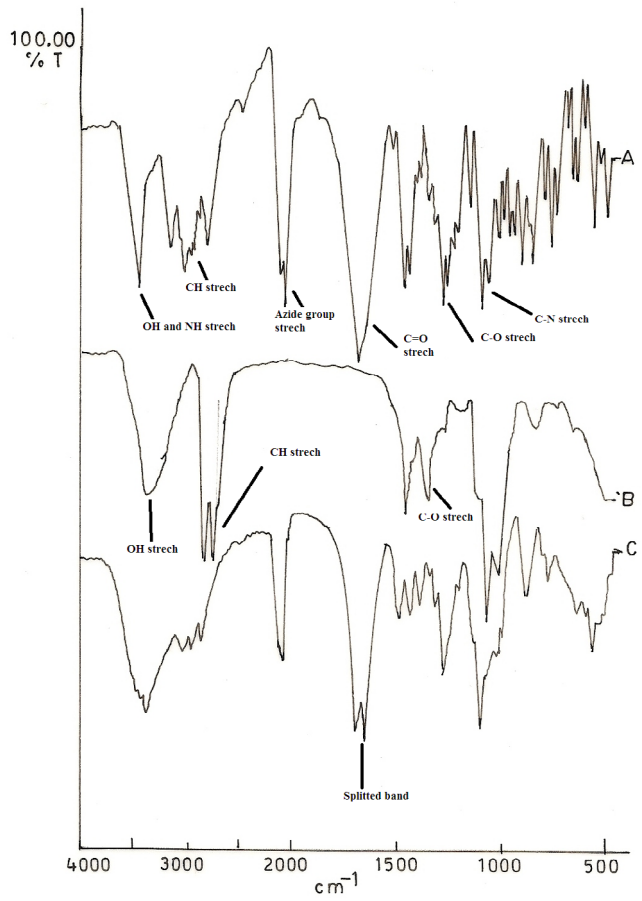

Figure 2: FTIR spectra of AZT (A), PETIM dendrimer (B) and AZT-dendrimer complex (C).

was 6.5 ± 0.3 % compared with 95.8 ± 4.1% from the control drug solution. AZT release was prolonged upto 14 h with AZT-dendrimer complex (94.3 ± 3.8%). Drug release from the AZT-dendrimer was steady and slow and decreased as a function of time. These observations are in accordance with the release profile of various drugs encapsulated in dendrimers [19].

Hemolytic toxicity of AZT-dendrimer complex

Table 2 summarized the results of hemolytic toxicity assay of PETIM dendrimer alone, AZT solution and AZT-dendrimer complex after 1 h of incubation at concentration of 20, 40 and 60 µg/ml. AZT solution in PBS showed hemolytic effects and at 40 µg/ml solution of AZT causes 90.2 ± 7.8% hemolysis. Complexation of AZT with dendrimer causes reduction of hemolytic effect of AZT and at 40 µg/ml only 39.0 ± 4.0% hemolysis was observed. This showed the protective effect of AZT-dendrimer complex probably due to sustained release of AZT from AZT-dendrimer complex. Dendrimer alone also showed the concentration dependent hemolytic effect (Table 2). Hemolytic toxicity is a well known disadvantage associated with dendrimer as carrier system due to its polycationic nature. PAMAM [Poly(amido amine)] dendrimer is well known for causing the hemolytic toxicity and reported 60% hemolysis at the concentration of 10 µg/ml [20]. In comparison developed AZT-dendrimer, complex showed only 38.18 ± 1.6% hemolysis at 6-fold higher concentration of 60 µg/ml. This is may be due to chemical nature of PETIM dendrimer. PAMAM dendrimer

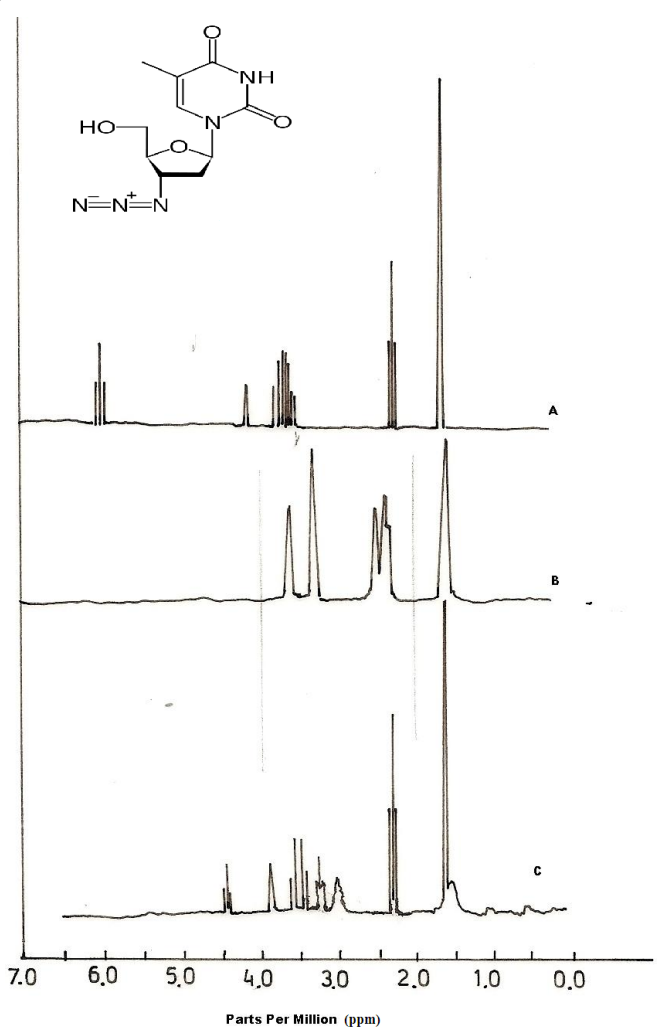

Figure 3: ^{1}H NMR spectra of AZT (A), PETIM dendrimer (B) and AZT-dendrimer complex (C) in D_2O.

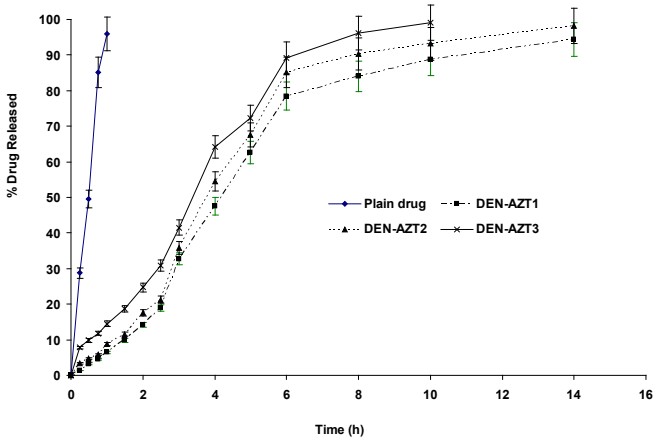

Figure 4: *In-vitro* drug release of AZT-dendrimer complex and plain drug across the dialysis membrane. Data are represented as Mean ± SD (n=3).

found to be hemolytic in number of studies due to its net positive charge on the surface and interacted with negatively charged surface of red blood cells and causes the aggregation and hemolysis of RBC_s [21]. In comparison, PETIM dendrimer has net negative charge on the surface that minimized the interaction with RBCs.

Morphological changes in the red blood cell shape in response to interactions with dendrimer were also studied by optical microscopy method. Figure 5 shows the photomicrograph of red blood suspension incubated with Phosphate buffer saline and saline that act as negative control, distilled water that cause 100% hemolysis used as positive control, dendrimer solution and AZT-dendrimer complex. Distilled water shows 100% cell lysis as evident by photomicrograph and phosphate buffer saline shows no hemolysis. Cells displayed the characteristic irregular contour due to the folding of the periphery. At a higher dendrimer concentration, cells elongated and spindle-shaped forms were observed. Reduction in the toxicity of AZT was observed due to the stable encapsulation in dendrimer.

Cytotoxicity assay

Cellular toxicity of AZT solution and AZT-dendrimer complex was investigated using MTT assay and results are summarized in Figure 6. Cytotoxicity assay was performed in concentration range of 4.68 µm to 0.58 µm with 2X dilution. In concentration range of 4.68, 2.34, 1.17 and 0.58 µm, AZT-dendrimer complex exhibited % cell viability as 26.9%, 74.8%, 96.8% and 99.12%, respectively. Comparative study was done with AZT free drug which showed 13.15%, 54.1%, 72.4% and 95.6% viability at same concentrations, respectively. The results of the cytotoxicity assay demonstrate that complexation of AZT with dendrimer leads to decrease in cytotoxicity of drug. The probable reasons for the reduced cytotoxicity of AZT-dendrimer complex are high entrapment efficiency, high serum stability and sustained release characteristic.

Pharmacokinetic and biodistribution studies

The biodistribution pattern of free drug, and AZT-dendrimer complex after 6 and 12 h administration have been shown in Figure 7. The pharmacokinetic parameters of AZT after administration in free from and as drug-dendrimer complex are shown in Table 3. The organ distribution pattern and plasma concentration of AZT in the case of AZT-dendrimer complex and free drug establish the superiority of AZT-dendrimer complex. The plasma level of AZT after application of AZT-dendrimer complex was found to be 4-fold higher after 6 hr and 18-fold higher after 12 hr of administration. The reason for significant less plasma concentration with plain drug solution is its very short biological half-life of AZT (0.9 hr). AZT is going to release in sustained manner with AZT-dendrimer complex and maintain the plasma concentration to 12 h. The results are in accordance with the similar findings of the study conducted by Wannachaiyasit et al. [9], in which Dextrin-Zidovudine conjugate found to have significant increase in the plasma half-life of AZT in comparison to free drug.

Drug-dendrimer complex significantly altered the tissue distribution of the AZT in comparison to plain drug solution. Contrary to the administration of free drug, drug levels remained in blood for a much longer time after its administration in the form of AZT-dendrimer complex, indicating the longer plasma half-life and sustained release characteristic of developed carrier system. Furthermore, the administration of AZT-dendrimer complex resulted in substantially higher accumulation of anti-HIV agent in spleen, liver

% Hemolysis					
S.No	Conc. µg/ml	Dist. Water	Dendrimer	AZT Solution	AZT-Dendrimer Complex
1	20	100%	12.5 ± 1.2	51.0 ± 4.2	17.0 ± 1.8
2	40	-	33.04 ± 2.8	90.15 ± 7.8	39.0 ± 4.0
3	60	-	41.4 ± 3.5	100%	45.2 ± 5.1

Data are represented as Mean ± SD (n=3)

Table 2: Hemolytic toxicity evaluation of AZT-dendrimer complex.

Formulation Code	AUC (µg.hr/ml)	C_{max} (µg/ml)	$T_{1/2}$ (hr)
AZT	13.4 ± 0.3	3.17 ± 0.2	1.9 ± 0.1
AZT-dendrimer complex	21.7 ± 0.8	3.29 ± 0.3	7.21 ± 0.4

Data are represented as Mean ± SD (n=3)
Abbreviations: AUC, area under the concentration–time curve C_{max}, peak plasma concentration; $T_{1/2}$ biological half life

Table 3: Pharmacokinetic parameters of AZT after administration in free form and as AZT-dendrimer complex.

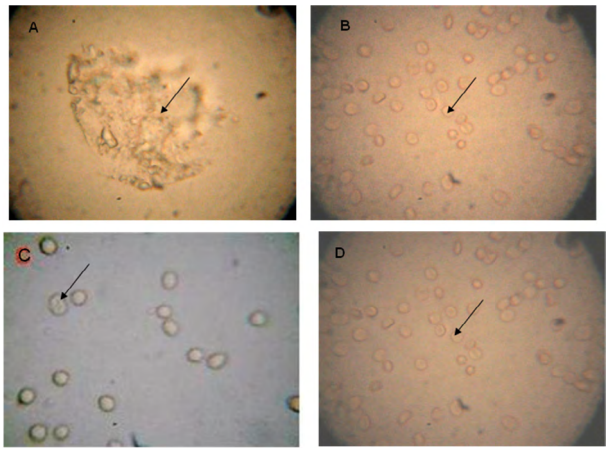

Figure 5: Optical microscopy photomicrographs of red blood cells incubated at 37 ± 1°C for 1 h with distilled water as positive control (A), Dendrimer solution (B), AZT solution (C) and AZT-dendrimer complex. (Magnification 450X).

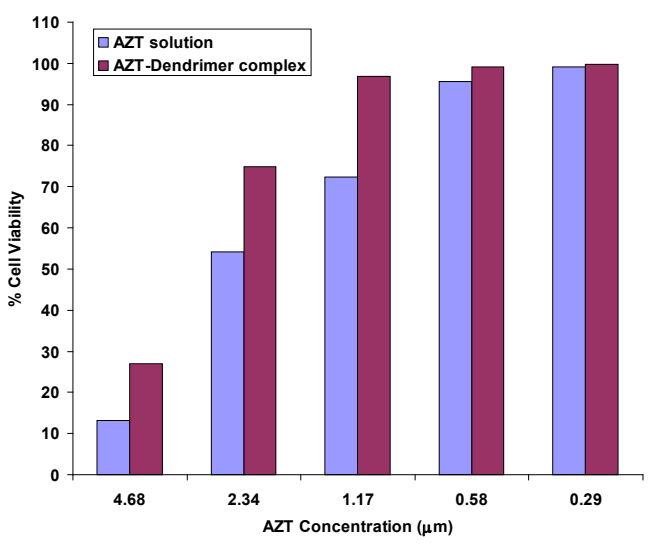

Figure 6: Comparative cytotoxicity of the AZT free drug and AZT-dendrimer complex after 24 h of incubation. Data are represented as Mean ± SD (n=3).

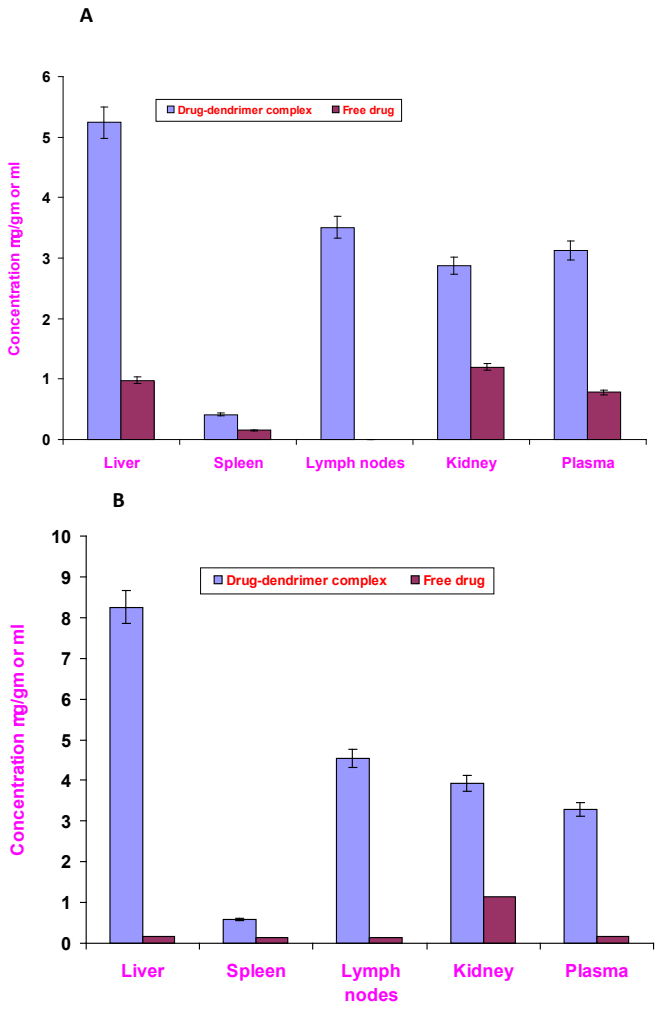

Figure 7: Biodistribution pattern of free drug, and AZT-dendrimer complex after 6 h (A) and 12 h (B) of administration. Data are represented as Mean ± SD (n=3).

and lymph nodes (Figure 7b). Results from these studies clearly indicate that a higher accumulation of AZT-dendrimer complex occurred in RES (reticuloendothelial system) tissue, compared with free drug. The reason for the higher concentration of AZT in RES organs is that the dendrimer formulation is preferentially taken by the cells of the RES organs [22]. A major limitation associated with the use of AZT is its dose dependent toxicity. The entrapment of drug into carrier system represents a potential approach for overcoming the toxicity by its selective uptake in RES, which are important hosts for HIV and play a key role in the pathogenesis of AIDS by providing long term reservoirs for the virus [23]. The use of dendrimer as drug carriers constitutes an attractive approach since they should concentrate the antiviral agents within cells susceptible to HIV infections, therefore improving the antiviral efficacy of the active agents and reducing their systemic toxicity.

Conclusion

AZT is an effective antiretroviral drug and results of the present study demonstrated that it can be effectively encapsulated in PETIM dendrimer for its sustained delivery. The percentage incorporation efficiency of AZT in AZT-PETIM dendrimer complex was found to be maximum at a ratio of 3 moles of AZT with 1 mole of PETIM dendrimer. The characterization of the prepared complex was done using FTIR and NMR analysis. PETIM dendrimer successfully entrapped the zidovudine, and *in-vitro* drug release study showed its sustained release potential. Reduction in the hemolytic toxicity of AZT was observed due to the stable encapsulation in dendrimer when compared to pure AZT drug solution. *In vivo* Pharmacokinetic study also demonstrated the sustained release characteristic of AZT-dendrimer complex. Biodistribution studies showed the accumulation of drug in RES organs. RES organs act as a host for HIV virus and virus replications take place in these organs. Targeting of AZT with dendrimer complex in RES organs would result in better pharmacodynamic of AZT for effective management of disease. The findings of the present study reveal that PETIM dendrimer is a better alternative for sustained drug delivery of zidovudine in comparison to present conventional therapy.

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